

Cite this: DOI: 10.1039/c0xx00000x

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Very Bright Europium Complexes that Stain Cellular Mitochondria

James W. Walton^b, Adrien Bourdolle^a, Stephen J. Butler^b, Marine Soulie^c, Martina Delbianco^b, Brian K. McMahon^b, Robert Pal^b, Horst Puschmann^b, Jurriaan M. Zwier^c, Laurent Lamarque^c, Olivier Maury^{*a}, Chantal Andraud^a and David Parker^{*b}

Received (in Cambridge, XXX) Xth July 2012, Accepted Xth XXXXXXXXXX 2012

DOI: 10.1039/b000000x

The synthesis, structure and photophysical properties of a series of highly emissive europium complexes is reported.

Certain complexes enter mammalian cells by macropinocytosis and stain the mitochondria selectively, allowing observation of the Eu emission *in cellulo* by time-gated spectral imaging.

Emissive lanthanide complexes for use as tags in bioassays or as optical probes require both a high emission quantum yield and large molar absorptivity at an excitation wavelength in the range 337 to 405 nm to give high brightness, B , where $B = \epsilon \cdot \phi$.¹ Using sensitised emission, the incorporation of multiple chromophores into a polydentate ligand has been studied, allowing efficient energy transfer to a bound Eu(III) ion that is efficiently shielded from vibrational deactivation by solvent.² In aqueous media, no 1:1 [Eu.L] systems have been reported with a brightness ($\lambda_{exc} > 337$ nm) exceeding $3000 \text{ M}^{-1} \text{ cm}^{-1}$.

Here, we report systems in which the brightness is an order of magnitude larger. Moreover, certain complexes are taken into mammalian cells, allowing their use in microscopic imaging. In designing these systems, we have combined the very effective shielding of the Eu(III) ion using nonadentate ligands based on triazacyclononane^{3,4} with strongly absorbing *p*-substituted aryl-alkynyl groups, [Eu.L¹⁻⁴].⁵ Both carboxylate and phosphinate substituted systems have been prepared⁶, and the synthetic pathway allows the preparation of derivatives that can be conjugated to a vector. In the phosphinate systems,³ the phosphorus substituents adopt a common configuration in the complex, and more effectively shield the excited Ln ion from intermolecular quenching processes.

The ligands and their Eu complexes were prepared using established methods.⁶ In the case of [Eu.L^{2c}], the third substituent, bearing a remote protected amine group, was introduced last, following stepwise alkylation and de-protection of mono-BOC-triazacyclononane⁶. Crystals of [Eu.L^{2a}] grew from aqueous methanol and the structure of [Eu.L^{2a}] revealed that the Eu ion is encapsulated by the ligand in a tricapped trigonal prismatic array (Figure 1). The nearest waters are over 6 Å from

the metal ion, and the complex is slightly distorted from C₃-symmetry. This distortion may be related to the presence of several disordered solvent molecules in the lattice.

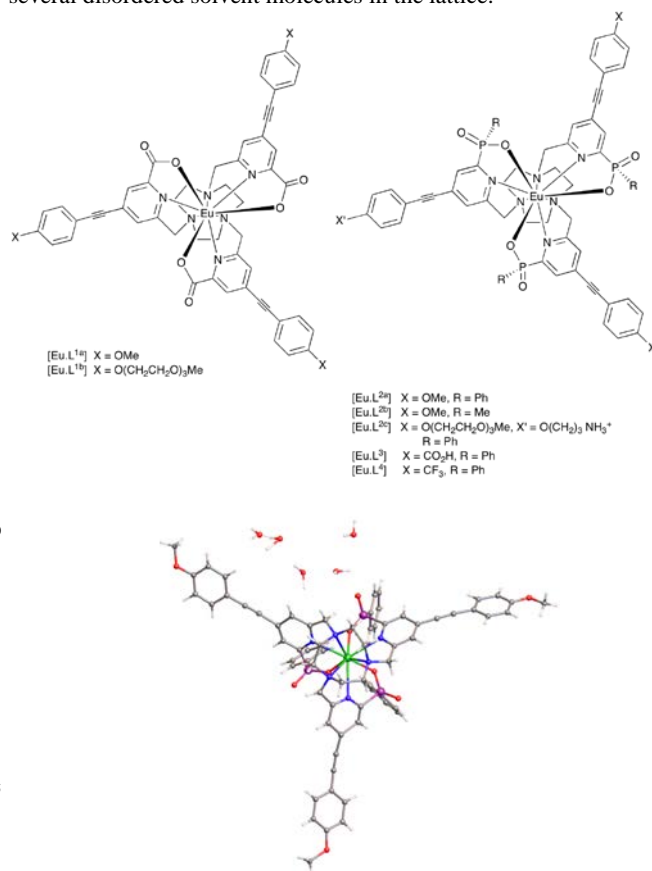


Figure 1 Molecular structure of the europium complex [Eu.L^{2a}] (120K) showing part of the hydration sphere; mean bond lengths (± 0.02 Å) are: Eu-N(ring) 2.68 Å; Eu-N(py) 2.66 Å; Eu-O 2.32 Å. Nearest waters are H-bonded to each P=O, with an average O-O distance of 2.66 Å; CCDC 857545.

This complex, in common with the other triphosphinate systems, exists as a 50:50 mixture of Δ -(SSS) and Λ -(RRR) isomers. In solution, one ^{31}P NMR resonance is observed; the paramagnetically shifted ^1H NMR spectra of each Eu complex are consistent with average C_3 -symmetry.

Table 1 Selected photophysical data^c for Eu(III) complexes (295K, MeOH, or as stated)

| Complex | $\lambda_{\text{max}}/\text{nm}$ | $\epsilon/\text{mM}^{-1}\text{cm}^{-1}$ | $\phi_{\text{em}}/\%$ | τ/ms |
|------------------------------------|----------------------------------|---|-----------------------|------------------|
| [Eu.L ^{1a}] | 338 | 58.0 | 48 | 0.95 |
| [Eu.L ^{1b}] | 338 | 55.0 | 25 | 1.06 |
| [Eu.L ^{2a}] | 332 | 58.0 | 52 | 1.30 |
| [Eu.L ^{2b}] ^a | 331 | 58.1 | 39 | 1.03 |
| [Eu.L ^{2c}] ^b | 332 | 60.0 | 54 | 1.00 |
| [Eu.L ³] | 322 | 59.2 | 37 | 1.24 |
| [Eu.L ⁴] | 307 | 63.5 | 15 | 1.35 |

^a in water; in MeOH $\phi_{\text{em}} = 43\%$ and $\tau = 1.18\text{ ms}$; ^b in 3:1 H₂O/MeOH; ^c errors on quantum yields and lifetimes are $\pm 15\%$; at 77K, phosphorescence spectra for [Gd.L^{1b}] and [Gd.L^{2b}] reveal a broad emission at 390 and 380nm respectively, consistent with a dominant charge transfer excitation band ^{5a}; ^d lifetime values in deuteriated solvents were typically 0.2 to 0.3 ms longer, e.g. for [Eu.L^{2c}], $\tau(\text{D}_2\text{O})$ was 1.28 ms, consistent with a metal solvation state, $q = 0$.

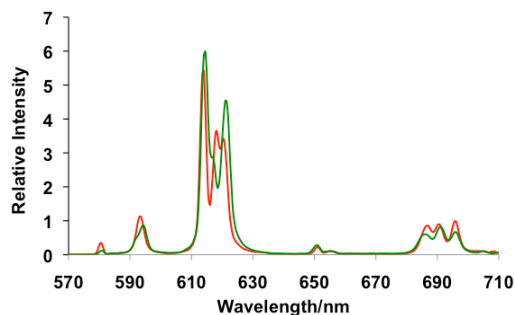


Figure 2: Europium emission spectra of [Eu.L^{1a}] (green) and [Eu.L^{2b}] (295K, H₂O, $\lambda_{\text{exc}} 355\text{ nm}$), showing minor changes in spectral form in the hypersensitive $\Delta J = 2$ manifold around 615 nm, associated with variation of oxygen donor polarisability.

Each chromophore absorbs around 310 to 340 nm, with an overall extinction coefficient of 55-60,000 $\text{M}^{-1}\text{cm}^{-1}$ (Table). Emission spectra in aqueous or methanol solutions were very similar for each Eu complex (Figure 2). The Eu complexes of L^{1a}, L^{2a}, L³ and L⁴ dissolved readily in MeOH, but were not soluble in water. The water solubility was higher with the complexes bearing PEG substituents, but did not allow greater than 2 μM solutions to be made up; for the PMe phosphinate complex, [Eu.L^{2b}], the limiting solubility was about 20 μM . A very intense set of $\Delta J = 2$ transitions was observed around 610-620 nm, and the spectral form was consistent with C_3 -symmetry. Overall emission quantum yields ranged from 15-54%, with the lower

values for the *p*-CF₃ substituted complex, [Eu.L⁴] presumably reflecting a less efficient intramolecular energy transfer step.

Following incubation of [Eu.L^{2b}] (10mM, 30 min) in the growth medium of NIH-3T3 (mouse skin fibroblasts), CHO (Chinese hamster ovarian) or PC-3 (human prostate cancer) cells, staining of the cell could be observed by confocal fluorescence microscopy. The cell images (Figure 3) revealed selective staining of the mitochondria, confirmed by co-localisation experiments with Mitotracker GreenTM, (ESI). Spectral imaging of washed cells, using time-gated methods, showed the characteristic europium spectral signature, confirming internalization of the intact complex. The europium complexes bearing PEG groups, were not internalized by live mammalian

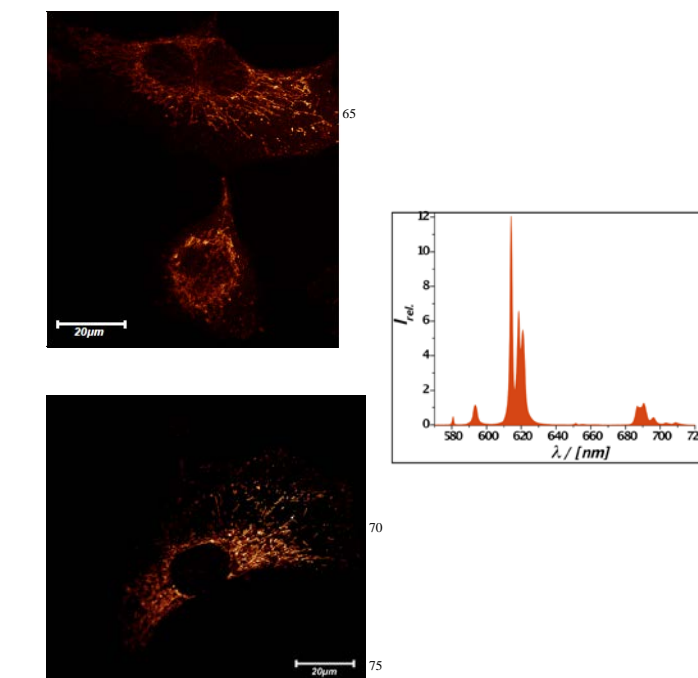
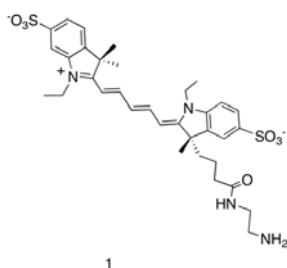


Figure 3: left: Bidirectional confocal microscopy images (Leica SP5 II) showing staining of cellular mitochondria in NIH 3T3 cells for [Eu.L^{2b}] (10 μM , 15 min loading time, $\lambda_{\text{exc}} 355\text{ nm}$, 100 Hz scan, hybrid detector 600-720 nm, 0.77 μm^3 voxel size, 12mW laser power, 40s image acquisition time); right: time-gated spectral image of a cell ($\lambda_{\text{exc}} 365\text{ nm}$, $t_{\text{int}} 2\text{ ms}$, $t_{\text{gate}} 10\mu\text{s}$, 10,000 scans averaged duty cycle using a 2D CCD detector, 100s acquisition time). The upper image shows a dividing cell (bar = 20 μM).

Cell uptake was inhibited (>30%) by pre-administration for 30 minutes with amiloride (3mM) or wortmannin (0.3 μM), or by lowering the temperature to 5 $^{\circ}\text{C}$. Such behaviour is consistent with cell uptake by macropinocytosis, as revealed recently for a wide range of emissive lanthanide complexes bearing heterocyclic sensitizing groups.⁷ The lack of uptake of [Eu.L^{1b}] and [Eu.L^{2c}] may reflect inhibition of a cell surface protein binding step that is essential for macropinocytosis. Using a standard MTT assay, assessing perturbation of mitochondrial redox activity, the IC₅₀ value was calculated to be >150 μM for [Eu.L^{2b}]. The amount of complex internalized was estimated by ICP-MS analysis of intracellular Eu, for a set of 390,000 washed and counted cells, revealing the complex concentration within the

cell to be $0.65(\pm 0.3)$ μM for a $10\mu\text{M}$ loading, under the stated conditions.⁸



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Furthermore, these Eu complexes can act as effective donors to near-IR acceptor dyes, such as the cyanine dye, **1**, allowing their use in time-resolved homogeneous FRET assays.⁹ A short comparative analysis of the efficiency of energy transfer was undertaken, comparing the behaviour of $[\text{Eu.L}^{1a}]$, $[\text{Eu.L}^{2a}]$ and $[\text{Eu.L}^{2b}]$ in MeOH and 1:1 aqueous methanol. The quenching of the Eu emission was monitored as a function of added dye concentration over the range 0.3 to $5\mu\text{M}$, using $5\mu\text{M}$ solutions of the Eu complex. The second order rate constants characterizing intermolecular energy transfer were 1.45, 0.57 and $0.65\text{ mM}^{-1}\text{s}^{-1}$ associated with Forster radii of 6.86, 6.95 and 6.76 nm respectively.¹⁰ The lower efficiency of quenching with each phosphinate complex may reflect both the slightly larger spectral overlap integral in $[\text{Eu.L}^{1a}]$ (Figure 3: $\Delta J = 2$ manifold is about 10% larger for $[\text{Eu.L}^{1a}]$ vs $[\text{Eu.L}^{2a/2b}]$), as well as the different electric dipole transition moments in the complexes.

In conclusion, the europium complexes defined here possess a brightness that lies in the range $15,000$ to $30,000\text{ M}^{-1}\text{ cm}^{-1}$ at 337 nm, auguring well for a role as donors in FRET bio-assays and as 2-photon probes.^{4a} An example is presented of cell uptake by macropinocytosis leading to selective mitochondrial staining; the very high brightness aids rapid spectral imaging. This behaviour paves the way for examination of analogues as intracellular optical probes, in which the emission spectral signature is a function of a biochemical variable, such as pH, bicarbonate or related bioactive species.¹¹ Furthermore, these examples highlight the opportunities to develop emissive metal coordination complexes as more general stains and probes for the biosciences.

We thank the EPSRC, ESF COST CM-1006 and the ERC (DP) for support (FCC 266804).

Notes and references

^a University of Lyon 1, ENS Lyon, CNRS - UMR 5182, 46 Allée d'Italie 69364 Lyon, France; E-mail : olivier.maury@ens-lyon.fr

^b Department of Chemistry, Durham University, South Road, Durham, DH1 3LE, UK. Fax: +44-191-3844737; Tel: +44-191-3342033; E-mail: david.parker@dur.ac.uk

^c Cisbio Bioassays, Parc Marcel Boiteux, BP 84175, 30200 Codolet, France

[†] Electronic Supplementary Information (ESI) available: Details of complex synthesis and characterization, microscopy/spectroscopy instrumentation and the structural analysis of $[\text{Eu.L}^{2a}]$ are available: CCDC 857545. For ESI and crystallographic data in CIF or other electronic format see DOI: 10.1039/b000000x/

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